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Functional Identification of Interneurons Responsible for Left-Right Coordination of Hindlimbs in Mammals

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Summary

Local neuronal networks that are responsible for walking are poorly characterized in mammals. Using an innovative approach to identify interneuron inputs onto motorneuron populations in a neonatal rodent spinal cord preparation, we have investigated the network responsible for left-right coordination of the hindlimbs. We demonstrate how commissural interneurons (CINs), whose axons traverse the midline to innervate contralateral neurons, are organized such that distinct flexor and extensor centers in the rostral lumbar spinal cord define activity in both flexor and extensor caudal motor pools. In addition, the nature of some connections are reconfigured on switching from rest to locomotion via a mechanism that might be associated with synaptic plasticity in the spinal cord. These results from identified pattern-generating interneurons demonstrate how interneuron populations create an effective network to underlie behavior in mammals.

Introduction

A major challenge for modern neuroscience is to unravel the neuronal networks underlying different types of behaviors, and in particular those of mammals that display a behavioral repertoire similar to humans. This analysis has been attempted for many higher, complex behaviors involving structures such as cerebral cortex, the basal ganglia, or the cerebellum. However, due to the large numbers of neurons involved and the complexity of the behavior, this has proved an immense and difficult task. It is therefore clearly advantageous to study a behavior that is simpler in nature and for which the underlying neuronal network is more easily characterized. Neuronal networks within the spinal cord underlying the production of locomotion belong to this category (Stein et al., 1997). Although these locomotor networks, otherwise known as central pattern generators (CPGs), have been characterized in detail in two aquatic vertebrates (Roberts, 2000; Grillner et al., 1998), relatively little is known about the pattern of neuronal connectivity of the corresponding networks in mammals (Hultborn et al., 1998). Recently, the development of rodent in vitro preparations (Kudo and Yamada, 1987a; Smith and Feldman, 1987; Jiang et al., 1999) has provided a new basis for unravelling the CPG networks controlling locomotion in mammals. Characterization of the rodent CPGs is particularly advantageous given the scope for genetic manipulation in the mouse (Yu and Bradley, 2001) and the resultant advances in understanding the genetic basis of spinal cord development (Jessell, 2000; Lee and Pfaff, 2001; Goulding et al., 2002). Thus, the rodent in vitro spinal cord preparation has the capability to reveal both molecular and physiological aspects of neural integration in a behavioral context (Sharma and Peng, 2001), although at present a major stumbling block is the identification of functional CPG interneuron subtypes.

The isolated spinal cord of the newborn rat produces rhythmic motor outputs (similar to that underlying locomotion in intact adults) when stimulated by a variety of neurotransmitter agonists (Kudo and Yamada, 1987a; Cazalets et al., 1992; Kiehn and Kjaerulff, 1996). This motor activity can be related at a basic level to alternation between left and right hindlimbs and between flexor and extensors in each limb. The circuits responsible for generating such activity are located in the ventromedial area of the lumbar spinal cord and include commissural interneurons (CINs) necessary for correct left-right coordination (Kjaerulff and Kiehn, 1996; Kiehn and Kjaerulff, 1998). The crossed axons of these neurons make them readily identifiable both anatomically (Tessier-Lavigne and Goodman, 1996; Kaprielian et al., 2001) and electrophysiologically (Butt et al., 2002), and hence they are an ideal starting point to unravel mammalian spinal cord neuronal networks. From previous studies we know that the CINs of the locomotor CPG constitute a heterogeneous population with respect to their projection patterns (Eide et al., 1999; Stokke et al., 2002) and firing properties (Butt et al., 2002). Here we show in one group of CINs, the descending CINs (dCINs), that this diversity in firing underlies the functional coordination of both extensor and flexor motor neurons through a mix of inhibition and excitation. These dCINs include mono- and polysynaptically projecting neurons as well as a group of dCINs whose function is modulated on initiation of locomotion and show long-term synaptic plasticity. Our study provides the first identification of interneurons that may play a role in generating coordinated walking in mammals. Moreover, it reveals a differential distribution of the flexor and extensor CPGs in the dorsoventral axis, which may be related to genetically determined patterning of interneurons (Jessell, 2000; Lee and Pfaff, 2001; Goulding et al., 2002).

Results

The data reported here are from neurons located in the ventromedial region of the lumbar spinal cord, an area that is necessary and sufficient for the production of locomotion in the neonatal rat and other mammals (Kiehn and Kjaerulff, 1998). This region contains four anatomically defined groups of CINs with distinct projection patterns: three classes of CIN that project more than one segment and have either ascending (*a*CIN), descending (*d*CIN), or bifurcating axons (*ad*CIN) and a

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fourth class of short-range, segmental CINs (sCIN). Here we concentrate on CINs that project caudally from the lumbar L2-L3 segment to the contralateral cL4-cL5 segment and includes both the anatomically defined bifurcating (*ad*CIN) and descending (*d*CIN) commissural interneurons. The basic firing and cellular properties of these neurons, which we term dCINs, have previously been characterized in detail (Butt et al., 2002).

A total of 154 neurons were recorded, of which 49 were identified as caudally projecting dCINs by the presence of an antidromic action potential during electrical stimulation of the contralateral cL5 hemicord (Figure 1A). Antidromic potentials persisted in the presence of the glutamatergic antagonists CNQX (20 μM) and AP-5 (30 μM) and were abolished by collision with an orthodromic action potential (results not shown) (Butt et al., 2002). To characterize the role of these identified interneurons in the mammalian CPG, we have related each recorded L2-L3 dCIN to its synaptic effect on target L4-L5 motor neurons on the contralateral side of the spinal cord and its preferred phase of firing during locomotor-like activity.

The Postsynaptic Effect of Spinal Interneurons onto Target Motor Neurons Can Be Determined by DC Ventral Root Recordings

Typically, synaptic effects from a given neuron onto a follower neuron are determined by dual intracellular recordings. This approach works nicely in a laminar structure like hippocampus and cortex, or when neurons can be visualized (Mason et al., 1991; Galarreta and Hestrin, 1999; Gibson et al., 1999). However, in the intact mammalian spinal cord, such dual recordings are more difficult and are bound to give a low yield. To bypass this obstacle, we have used a method reliant on dCIN spike-triggered averaging of DC ventral root recording (Figures 1A and 1B; also M. Raastad and O.K., unpublished data; M. Raasted and O.K., 1995, Soc. Neurosci., abstract). This method is similar to that used previously in chick embryos to test the synaptic effect of Renshaw cells on motor neurons (Wenner and O'Donovan, 1999; Wenner et al., 2000) and the used sucrose gap recording method by Jankowska and colleagues (Brink et al., 1981, 1983). The DC signal recorded in close proximity to the exit point of the cL4 rootlets is analogous to recording an attenuated intracellular potential of all the motor neurons exiting via that root. If the dCIN was silent, spikes were elicited by depolarizing current injection. Initial analysis of the spike-triggered averaged DC potentials were conducted in the absence of locomotor-inducing drugs.

Using this method, we found that constant latency depolarizing or hyperpolarizing responses were present in 46 out of 49 of the dCIN spike-triggered averages of the cL4 DC signal and in only 9 of the 105 unidentified neurons; the other 96 unidentified neurons evoked no detectable signal in the averaged cL4 DC trace. The data from the 9 unidentified neurons were excluded from this study although they might represent short-range CINs whose axons only project as far as cL4 and therefore would not be detected by antidromic stimulation of the contralateral L5 segment.

To confirm that the DC potentials were synaptic in origin, extracellular calcium was lowered (n = 3) (Figure 1C). Perfusion with low-calcium Ringer's solution reversibly reduced the amplitude of the averaged signal (also M. Raastad and O.K., unpublished data). In addition, a negative correlation was observed in all tested cells between dCIN spike frequency, varied by constant intracellular current injection, and the cL4 DC signal amplitude (Figures 1D and 1E). Thus, at higher firing frequencies (>5 Hz) there was a strong depression of the DC signal with longer latency, putative polysynaptic signals (black symbols, Figure 1E), exhibiting more pronounced depression at low firing frequencies than short

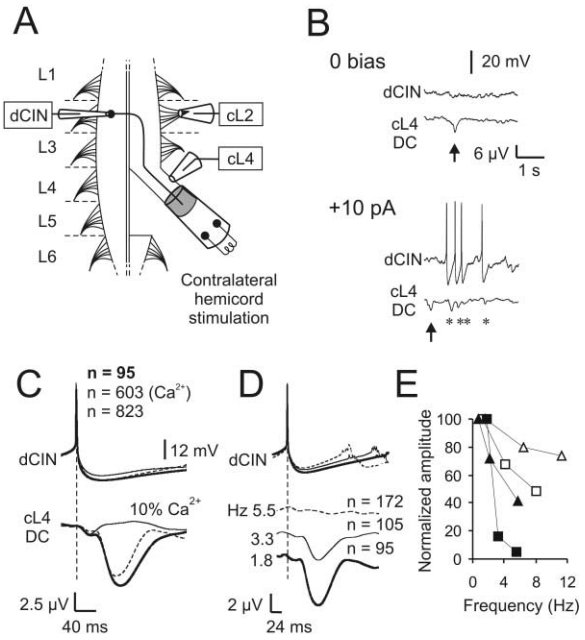

Figure 1. dCIN Spike-Triggered Average of cL4 DC Ventral Root Recordings Is a Measure of Synaptic Input onto Target Contralateral Motor Neurons in the Isolated Neonatal Rat Spinal Cord Preparation

(A) Experimental set-up with ventral surface up as viewed in the recording chamber. Commissural interneurons (dCIN) were identified by antidromic stimulation of the contralateral L5 hemicord. AC ventral root recording (cL2) was used to monitor rhythmic locomotor-like activity and postsynaptic effects of dCIN determined by DC ventral root recording of the L4 (cL4) contralateral to the site of intracellular recording.

(B) Individual deflections were observed in the raw cL4 DC trace in the absence of dCIN spiking (arrowheads, top and bottom panels) recorded at rest. In the example shown, current injection (bottom panel, +10 pA) resulted in dCIN firing, which was associated with a signal in the DC recording as indicated by the asterisks below the trace.

(C) Reducing the extracellular Ca^{2+} concentration resulted in suppression (thin line; n = 603 spikes) of the control spike-triggered average DC signal (bold line; n = 95 spikes) as well as a reduction in the dCIN spike after-hyperpolarization. Washout with normal Ringer's resulted in a recovery of the signal (dashed line; n = 823 spikes).

(D) Progressively increasing dCIN spike frequency by current injection in the absence of locomotor-inducing drugs resulted in failure of the long latency DC signal at 5.5 Hz (dashed line).

(E) Normalized data from four dCINs: two polysynaptic inhibitory dCINs (black symbols), which showed strong depression at low firing frequencies and short latency inhibitory (white squares) and excitatory (white triangles) dCINs in which the signals persisted beyond 5Hz (average of >50 spikes for each data point).

latency, putative monosynaptic signals (white symbols). This might reflect temporal summation of evoked postsynaptic potentials or a frequency-dependent synaptic depression that is a pronounced phenomenon in the neonatal rat spinal cord and appears at relatively low frequencies (Lev-Tov and Pinco, 1992). The firing frequency range where it becomes apparent in CINs is well above the instantaneous firing frequency recorded from spinal interneurons during locomotion (Raastad and Kiehn, 2000; Tresch and Kiehn, 1999; Butt et al., 2002). In addition to these tests, the synaptic nature of the signal was tested by application of appropriate neurotransmitter antagonists (see below), and data presented here includes only dCIN inputs onto motor neurons in which the pharmacology of the postsynaptic effect was confirmed.

Four Categories of dCINs Based on Their Postsynaptic Effects

After determining the synaptic sign of dCIN connections onto cL4 motor neurons, the pharmacology of these connections was tested in the nonlocomoting, quiescent spinal cord, either prior to or 30–40 min after N-methyl-D-aspartate- (NMDA) and serotonin (5-HT)-induced locomotion had been reversed by washing out with normal Ringer's. The quiescent "rest" state was typified by a lack of cL2 ventral root activity and no rhythmic or large scale oscillations in the cL4 DC trace. In order to compensate for the tonic depolarization induced by antagonists for inhibitory neurotransmitters, the level of steady bias current injection was varied in order to maintain a stable firing frequency (see Experimental Procedures).

Based on their pharmacology and sign of synaptic drive to contralateral L4 motor neurons, we found three categories of dCIN that had the same postsynaptic effects at rest and during locomotion: (1) monosynaptic excitatory dCINs, (2) monosynaptic inhibitory dCINs, and (3) dCINs that inhibited contralateral motor neurons via polysynaptic pathways. The monosynaptic excitatory dCINs (Figure 2A) evoked depolarizing responses in the cL4 DC recording at short latencies (6.5–13.9 ms measured from the peak of the averaged intracellular dCIN spike; Figure 2D). These latencies are in a comparable range to monosynaptically evoked ventral root potentials (Kudo and Yamada, 1987b). They were sensitive to a combination of CNQX (20 μM) and AP-5 (30 μM) ($n = 9$) and persisted in the presence of the barbiturate mephenesin (1 mM) ($n = 2$), which has been shown to attenuate or block polysynaptic transmission in the mammalian spinal cord (Lev-Tov and Pinco, 1992). An estimate of the synaptic delay derived from the conduction of the dCINs and the latency of the DC signal (see Experimental Procedures) was 3.3 ± 0.5 ms ($n = 9$). Monosynaptic inhibitory dCINs (Figure 2B) had short latency (6.2–13.7 ms; Figure 2E) hyperpolarizing responses that persisted in the presence of the glutamatergic agonists CNQX and AP-5 ($n = 8$) but were sensitive to the glycine antagonist strychnine (0.3 μM) but not the $GABA_A$ antagonist bicuculline (2.0 μM) ($n = 5$). dCINs that inhibited contralateral motor neurons via presumed polysynaptic pathways (Figure 2C) had long latency inhibitory (20.8–46.7 ms; Figure 2F) responses that were blocked by CNQX and AP-5, consistent with polysynaptic transmission via an intervening excitatory synapse ($n = 7$). In addition, the responses were reversibly abolished by the $GABA_A$ antagonist bicuculline ($n = 3$) but not by the glycinergic antagonist strychnine ($n = 2$).

The three populations of dCINs described above had the same synaptic sign at rest as during locomotor-like activity evoked by NMDA and 5-HT (see below). A fourth population of dCINs ($n = 11$) exhibited differing synaptic signs at rest and during drug-induced locomotor-like activity (Figure 3A). These neurons termed "switch cells" inhibited motor neurons via a GABAergic polysynaptic pathway during rest (latency 16.4–45.3), but on perfusion with either NMDA and 5-HT or 5-HT alone, they switched to exerting a monosynaptic excitation (latency 6.0–9.2 ms; Figure 3C). Application of bicuculline (Figure 3B) blocked the long latency inhibition observed at rest, resulting in the emergence of the short latency excitatory response ($n = 4$). This short latency excitatory response was not observed at rest unless we antagonized GABAergic transmission. Perfusion with CNQX and AP-5 abolished both types of signal in the DC trace, consistent with a polysynaptic pathway mediated via an excitatory synapse (Figure 3B) ($n = 11$).

The time course of the switch was followed in seven preparations in the presence of NMDA and 5-HT (Figure 3D) or 5-HT alone, at a concentration that did not elicit locomotion (≤ 7 μM) (Figure 3E). In all cases the switch in synaptic sign became apparent shortly after the drugs started to wash into the recording chamber (<120 s) and preceded the onset of organized locomotor-like activity. In five of the seven cells, when NMDA was coapplied with 5-HT to induce locomotor-like activity, the switch did not reverse immediately on washout of the drugs (Figure 3D) but persisted for 40 min or longer. In the presence of 5-HT alone, the switch was still observed in the absence of rhythmic ventral root bursting, but in all cases ($n = 7$), the sign of the synaptic input reversed rapidly on washout of 5-HT (Figure 3E). This suggests a reversal in sign of interneuron input between rest and locomotor-like states in some dCIN subpopulations, which is subject to long-term modulation.

In summary, based on their postsynaptic effects, we have defined four populations of dCINs: two populations with monosynaptically projecting excitatory or inhibitory effects, one polysynaptic inhibitory population, and a population of dCINs whose function switches from polysynaptic inhibition during rest to monosynaptic excitation during locomotion.

Preferred Phase of Firing during Locomotion for the Four Populations of dCINs

In order to begin to understand the function of the different dCIN subclasses, it is necessary to know when they are active during locomotion. We therefore determined the preferred phase of firing of the four populations of dCINs during stable locomotor-like activity induced by perfusion of the isolated spinal cord with NMDA (5–8 μM) and 5-HT (5–10 μM) (Figures 4A and 4B). Locomotor-like activity was seen as rhythmic bursts in the ventral root contralateral to the recorded interneuron (cL2, upper AC traces) and as oscillations in the contralateral L4 DC

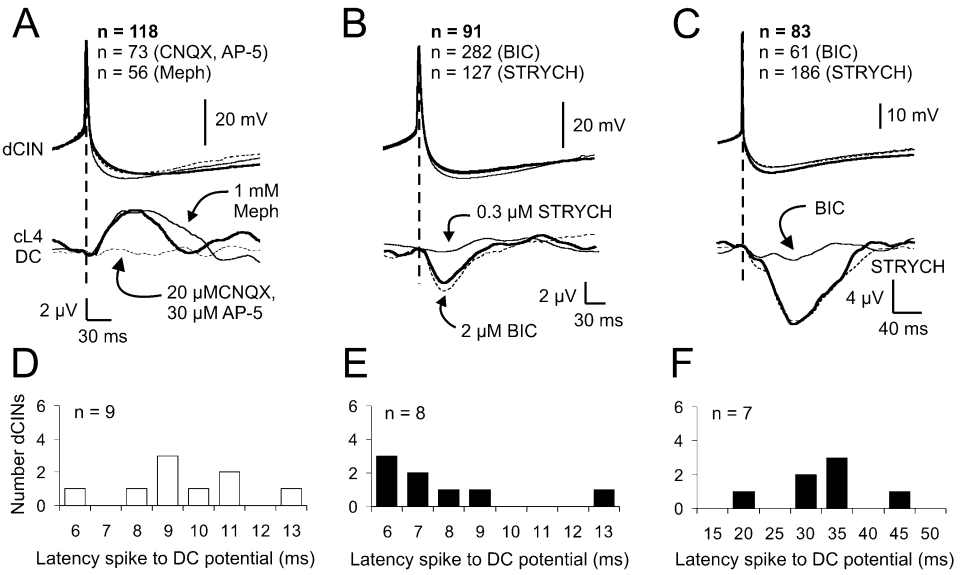

Figure 2. The Pharmacological Profile of the Postsynaptic Effect at Rest Define Three Groups of dCIN

(A) Monosynaptic glutametergic CINs had short latency excitatory responses that was blocked by CNQX (20 μM) in combination with AP-5 (30 μM) (dashed line), but not by prolonged incubation with mephenesin (thin line).

(B) Monosynaptic glycinergic CINs had short latency inhibitory responses that were sensitive to strychnine (0.3 μM) (thin line) and persisted in bicuculline (2 μM) (BIC) (dashed line).

(C) A third category of CINs showed GABAergic polysynaptic inhibition that were sensitive to low concentrations of bicuculline (BIC; thin line) or a combination of CNQX and AP-5 (not shown), but signals persisted in the presence of the glycinergic antagonist strychnine (STRYCH; dashed line).

(D–F) Histograms showing the latencies of the DC potentials calculated from the peak of the dCIN spike for excitatory (D), monosynaptic inhibitory (E), and polysynaptic inhibitory dCINs (F).

suction electrode recordings (cL4, lower traces). Since the ipsilateral L2 is strictly alternating with the contralateral L2, we will for convenience relate the firing of the dCINs to the ipsilateral phase. Furthermore, the dominant burst in L2 corresponds to flexor activity, which is active out of phase with the predominantly extensor activity in L5 (Kiehn and Kjaerulff, 1996). dCINs that fired in phase with the ipsilateral L2 burst, out of phase cL2, are therefore named *flexor-related dCINs* (Figure 4A; gray-shaded area). In contrast, those firing out of phase with iL2 and in iL5 phase are named *extensor-related dCINs* (Figure 4B; unshaded area). Circular statistics (see Experimental Procedures) were used to determine the degree of rhythmicity and the preferred phase of firing. Pooled data in the form of histograms (Figure 4C) revealed that excitatory and inhibitory dCINs had preferred phases of firing in both flexor (iL2/cL5) and extensor phases (iL5/cL2). Figures 4D–4G show the preferred phases of firing and degree of rhythmicity represented as vector points for the excitatory and inhibitory populations when split into the four dCIN categories defined in the previous section. The position of the point in the circular plot determines the phase, while the distance from the center signifies the degree of rhythmicity. dCINs display all degrees of rhythmicity similar to what has previously been reported (Butt et al., 2002). The switch cells (white squares, Figure 4D) and polysynaptic inhibitory dCINs (black squares, Figure 4E) showed similar distributions in their preferred phases of firing, with both types of neurons firing in the ipsilateral flexor phase (shaded area) with the exception of one switch cell. Monosynaptic excitatory (white triangles, Figure 4F) and inhibitory dCINs (black triangles, Figure 4G) fired in both the ipsilateral extensor and flexor phases.

This organization suggests that contralateral L4-L5 motor neurons activity is coordinated with the ipsilateral L2 segment activity via both excitatory and inhibitory dCINs.

Functional Role of the Different Categories of dCINs in the Locomotor Network

From analysis of the postsynaptic effect and the phase relationship, described by the circular statistics, it is not immediately apparent what role the dCINs subserve to coordinate left-right activity in the caudal parts of the hindlimb CPG given that they mediate both inhibition and excitation throughout the locomotor cycle. These dCINs project axons from the predominantly flexor L2 to the contralateral, predominantly extensor L5; however, we have monitored their effects on cL4 motor neurons. Motor axons exiting via the L4 ventral root innervate both flexor and extensor muscles, as do L2 and L5, albeit to lesser extents (Nicolopoulos-Stournaras and Iles, 1983). Ventral root recordings of L4 can often be biphasic, with bursts of activity in both the predominantly flexor L2 and predominantly extensor L5 phases (Cazalets et al., 1992, and others). Alternatively, in some preparations the bursts are either predominantly extensor or flexor related.

Thus, we can envisage four potential types of connection between iL2 dCINs and target motor neurons in L4 during normal walking: in-phase crossed coordination

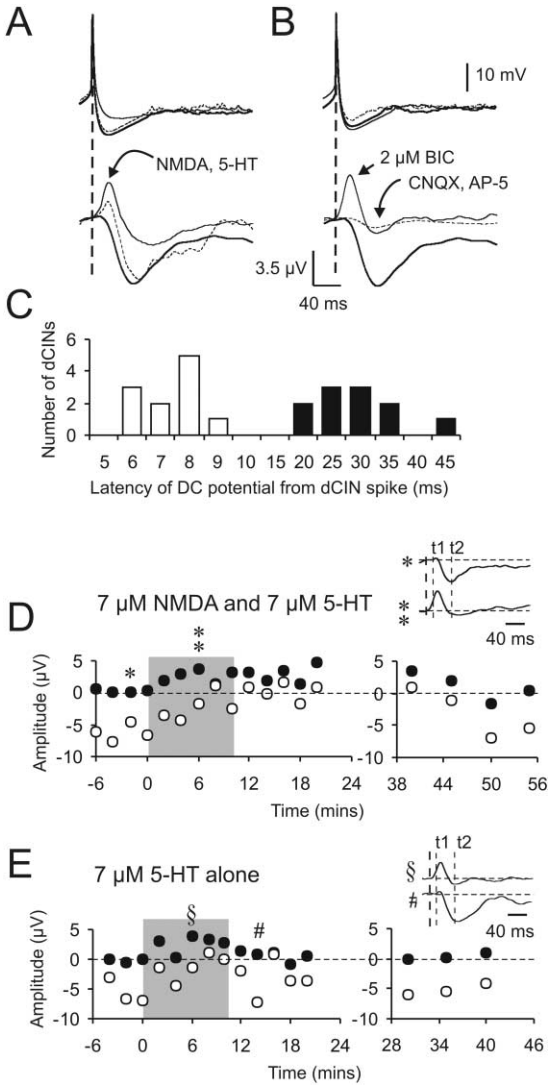

Figure 3. Switch Cells: A Fourth Category of dCIN Input

(A) Input from switch cells onto cL4 motor neurons alters from being polysynaptic inhibitory (bold line; n = 195 spikes) at rest to short latency excitatory (thin line; n = 182) during NMDA- (7 μM) and 5-HT- (7 μM) induced locomotor-like activity. The excitatory response persisted after drug washout (30 min wash; dashed line; n = 53). Perfusion with bicuculline (B) to block the polysynaptic inhibition results in the emergence of the excitatory input (thin line; n = 207). CNQX and AP-5 block all input from switch cells onto contralateral motor neurons (dashed line; n = 134). (C) Histogram showing the distribution of the latencies of the polysynaptic inhibitory responses recorded at rest (black bars) and the monosynaptic excitatory potentials recorded during locomotor-like activity (white bars). The time course of the switch was tracked during NMDA and 5-HT perfusion (D) and 7 μM 5-HT alone (E). Gray-shaded areas represent the 10 min period during which the drugs were applied. In the example shown, stable locomotor-like activity was evident after 6 min perfusion with NMDA and 5-HT. The switch to monosynaptic excitation in the presence of NMDA and 5-HT outlasted the initial 20 min recording time and only reversed after 40 min wash (right graph). Sample average DC traces as indicated by the symbols are shown as inserts with the time of the short latency (t1; black circles) and polysynaptic (t2; white circles) potentials indicated relative to that of the dCIN spike (first vertical dashed line).

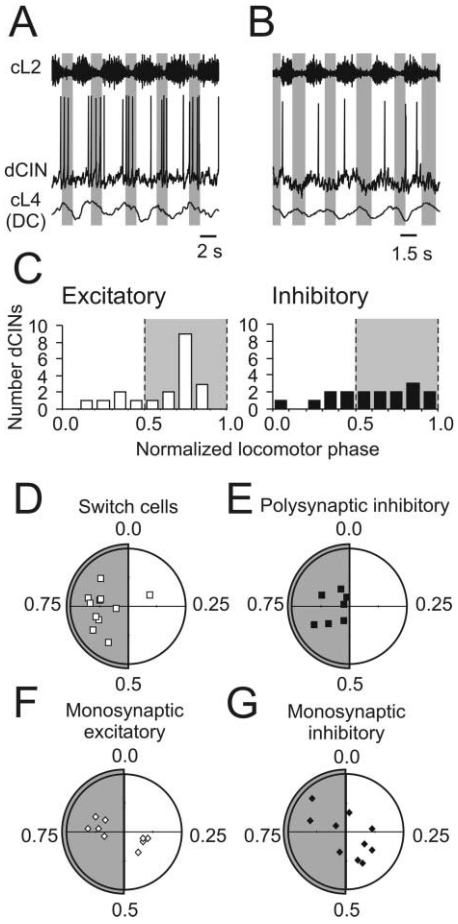

Figure 4. Firing Phase of dCIN Subtypes during Locomotor-like Activity

(A and B) Examples of rhythmic dCINs that fired in ipsilateral L2 flexor phase (gray shaded) (A) or ipsilateral L5 extensor phase (B) (contralateral L2 (cL2) phase; unshaded region). In all subsequent figures, gray-shaded areas represent the ipsilateral L2 flexor phase, which coincides with the contralateral L5 extensor phase, and vice versa for the unshaded area. Data from excitatory dCINs are white, whereas inhibitory dCIN data are black.

(C) Pooled data for excitatory (left panel) and inhibitory (right panel) dCINs showing the distribution of preferred phases of firing over a normalized locomotor cycle. Overall, inhibitory dCINs and excitatory dCINs fired in both the extensor and flexor phases.

(D–G) Plots of the dCIN vector points derived from the circular statistic categorized according to their postsynaptic effect on L4 motor neurons. Each point represents the vector derived from 25 spikes analyzed during stable locomotor-like activity. The direction of the vector represents the preferred phase of firing of the neuron, and the distance from the center point indicates the tuning of the spikes around their mean.

between (1) iL2 flexor (*f*)-related dCINs and cL4 extensor (E) motor neurons and (2) iL2 extensor(*e*)-related dCIN and cL4 flexor (F) motor neurons mediated by activity in excitatory dCINs; and out-of-phase activity between (3) iL2 extensor-related dCINs and cL4 extensor motor neurons and (4) iL2 flexor-related dCIN and cL4 flexor motor neurons mediated by inhibitory dCINs.

Support for this connectivity scheme comes from the analysis of the dCIN activity in preparation where the dominant L4 burst was either flexor or extensor related.

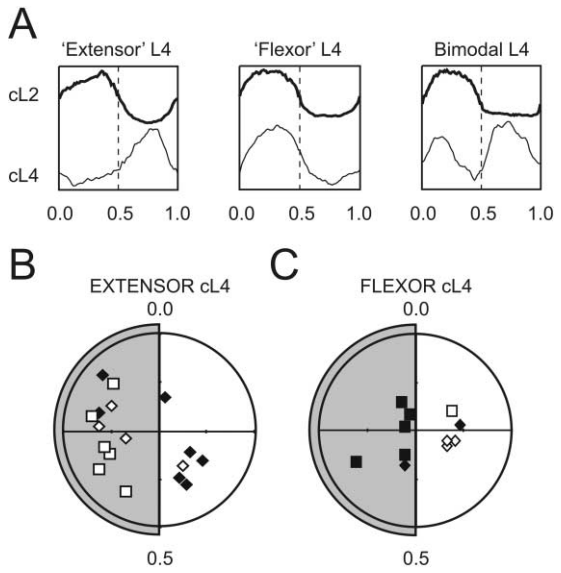

Figure 5. Excitatory and Inhibitory dCINs Define Activity in Both Flexor and Extensor L4 Motor Pools

(A) The peak phase of cL4 motor neuron activity varied between preparations relative to the predominantly flexor cL2 burst. Average cL4 DC oscillations (n = 50 for each preparation; thin line) relative to the rectified cL2 (bold line) AC locomotor-like bursts for typical extensor, flexor, and bimodal preparations.

(B and C) Breakdown of dCIN circular plot vectors according to target cL4 motor neuron type.

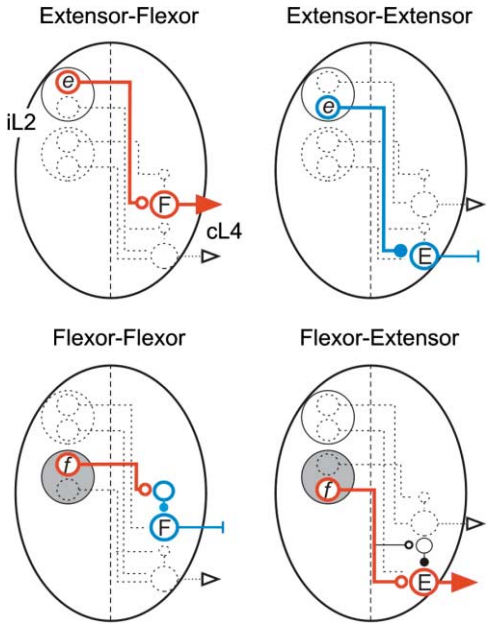

Figure 6. Schematics of the Principle Network Connections Coordinating Crossed L2-L4 Activity

Ipsilateral L2 extensor (*e*) and flexor (*f*) centers connect to both cL4 flexor (F) and extensor (E) motor pools. Excitatory synapses are represented by open red circles, inhibitory connections by filled blue circles. The polysynaptic inhibitory pathway modulated by 5-HT mediating flexor-extensor connections is shown in black. For clarity, nonactive pathways are shown as dashed lines.

These preparations allowed us to investigate the distribution of dCIN input onto flexor and extensor motor neurons in comparative separation (see Discussion). Oscillations of the cL4 DC recording were averaged relative to the predominantly flexor cL2 root (Figure 5A). Of the 37 preparations, 17 had cL4 roots out of the phase with the cL2, which we termed "extensor" cL4; 11 were in phase with cL2 and therefore represented dominant "flexor" activity; the activity in the nine remaining preparations exhibited clear bimodal activity. The phase of the L4 motor neurons relative to L2 did not shift in individual animals during the course of the experiments.

The distribution of dCINs present in extensor and flexor cL4 preparations revealed that in both situations there were inhibitory and excitatory inputs onto the motor neurons (Figures 5B and 5C). What is interesting, however, is the differential firing phases of dCIN types in the L4 extensor as opposed to flexor preparations. In the extensor cL4 preparations (Figure 5B), the majority of excitatory cells are switch cells (white squares) that fire in the iL2 flexor phase (gray-shaded semicircle), and the majority of inhibitory cells are monosynaptic (black diamonds) that fire in the iL2 extensor phase (nonshaded area). These cells will cause alternating excitation and inhibition of cL4 extensor-related motor neurons with the right timing during walking (Figure 6). In predominantly flexor cL4 preparations (Figure 5C), polysynaptic inhibitory dCINs cells (black squares) fire in the iL2 flexor phase (gray-shaded semicircle), while most of the cells firing in the iL2 extensor phase (nonshaded area) are last-order excitatory interneurons (white diamonds). These cells cause crossed coordination between the iL2 extensor-related CPG elements and cL4 flexor motor neurons (Figure 6). All four types of dCIN were found in bimodal preparations: the main crossed innervation of mixed flexor and extensor cL4 motor neurons being mediated by switch cells (n = 4) with lesser components consisting of pure monosynaptic excitation (n = 2) and mono- (n = 1) and polysynaptic inhibition (n = 2).

The differential pattern of the dCIN activity in flexor and extensor cL4 preparations suggests a contributing role for these neurons in shaping the final locomotor pattern (see Discussion).

The Flexor and Extensor CPGs Occupy Distinct Locations in the L2 Lumbar Segment

The position of the four different groups of dCINs was mapped in the transverse plane of the lumbar spinal cord as previously described (Figure 7A; Butt et al., 2002). Taking into consideration the possible role of the different categories of dCINs in the locomotor network (see previous section), it is evident from the pooled data that there is a segregation of flexor- and extensor-related dCINs (Figure 7B). Thus, the flexor-related dCINs (gray histogram bars), whether they are mediating excitation to L4 extensor motor neurons or inhibition to L4 flexor motor neurons, are present in greater numbers toward the ventral surface. The reverse pattern is seen for extensor-related dCINs (white histogram bars). We tested the null hypothesis that the ventrodorsal distribution of flexor and extensor-related L2 dCIN were the same. The null hypothesis was rejected at the 5% significance level (Mann-Whitney test, $0.01 < p < 0.02$) (Samuels, 1991).

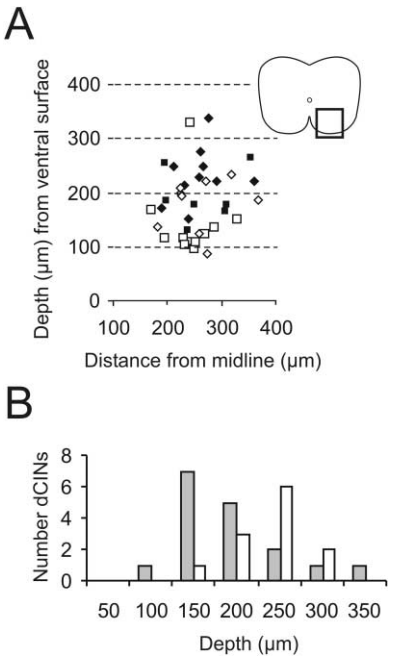

Figure 7. Anatomical Distribution of Physiologically Distinct L2 Flexor- and Extensor-Related dCIN Centers

(A) Plot of the location of switch cells (white squares), polysynaptic inhibitory (black squares), monosynaptic inhibitory (black diamonds), and excitatory (white diamonds) dCINs in the transverse plane of the ventromedial lumbar cord (see inset for location).

(B) Differential distribution represented as bar histograms of ipsilateral flexor- (gray bars) and extensor- (white bars) related L2 dCIN centers in the ventral-dorsal axis.

This finding leads us to suggest that the flexor and extensor CPGs in L2 are differentially distributed in the transverse plane.

Discussion

This study provides the first detailed characterization of identified neurons that are components of the rodent locomotor CPG. By focusing on distinct populations of commissural interneurons that are readily identified anatomically and electrophysiologically (Eide et al., 1999; Stokke et al., 2002; Butt et al., 2002), we have been able to deduce the role of these neurons in the CPG network by quantifying their rhythmicity and characterizing their postsynaptic effects.

We conclude from our studies that crossed coordination of the mammalian hindlimb locomotor network is most likely mediated by both inhibitory and excitatory components that originate from anatomically distinct but overlapping flexor and extensor centers in the ipsilateral L2 hemicord. We demonstrate that these interneurons form connections onto contralateral motor neurons via both monosynaptic and polysynaptic pathways, although excitatory connections are exclusively monosynaptic, and between crossed flexor-extensor or extensor-flexor centers only. These findings have general implications for our understanding of spinal cord function and development.

Methodological Considerations

A key element in our study has been the use of DC recordings from the ventral root to reveal the postsynaptic effects of identified interneurons. DC ventral root recordings from close proximity to the exit point of the nerves represent an attenuated version of the sum intracellular potential of the motor neurons exiting via that root. While DC recordings of ventral root activity in the spinal cord have previously been used to investigate *en* block motor neuron activity during locomotion or reflex activity (Bracci et al., 1998; Kudo and Yamada, 1987b; Tresch and Kiehn, 2000), this is the first time it has been used in combination with spike-triggered averaging to detect the functional input of individual locomotor-related interneurons onto target motor neurons in the quiescent and active in vitro mammalian spinal cord. The technique is similar to that used previously in chick embryos (Wenner and O'Donovan, 1999; Wenner et al., 2000), where it was used to determine the postsynaptic effect of putative Renshaw cells onto segmental motor neurons. A great advantage of this technique is its sensitivity: relatively few spikes are required to reveal postsynaptic potentials, even for polysynaptic connections. Moreover, when applied in vitro, pharmacological tests can easily be performed to confirm the synaptic nature of the signals. Thus, we have tested the sensitivity of the averaged DC trace to extracellular calcium concentrations in addition to showing that the signal shows a strong frequency-dependent depression similar to other synapses in the neonatal rat spinal cord (Lev-Tov and Pinco, 1992; M. Raastad and O.K., unpublished data). Furthermore, we also demonstrated that the DC potentials are sensitive to known neurotransmitter antagonists in a reversible manner. Based on these tests, we conclude that the DC signal does indeed represent synaptic potentials.

It is likely that these potentials result from dCIN inputs onto motor neurons. (1) DC potentials could also be recorded when the electrode tip was electrically insulated from the cord surface (see Experimental Procedures), excluding the possibility that the signals arise from fibers located in the adjacent cord. (2) The potentials were time-locked to the interneuron spike activity, which could be varied by current injection. (3) Moreover, it would appear that the method is highly selective given that we consistently detected synaptic potential from identified dCINs that are known to project caudally to the cL4 motor neurons area, but not from the large majority of recorded non-dCINs.

There is the possibility when spike-triggered averaging the DC trace in the active cord that the averaged signal is the result of or contaminated by a common locomotor drive onto both the recorded interneuron and motor neurons. However, there are a number of lines of evidence that suggest that the DC potentials detected represent a true reflection of the dCIN activity irrespective of whether the cord is active or not. First, with the exception of switch cells, DC potentials observed in the quiescent cords where locomotor drive is not a major factor were conserved in the active state. Second, our data include a number of nonrhythmic dCINs that fired throughout the locomotor cycle and yet still displayed constant DC potentials during locomotor-like activity irrespective of L4 phase. Third, not all rhythmic neurons

were as highly tuned as those shown in Figure 4 and often spikes appeared in the "wrong" phase. Selectively averaging these aberrant spikes did not alter the sign or latency of the DC signal (see Supplemental Figure S1A at http://www.neuron.org/cgi/content/full/39/1/953/DC1). Fourth, injecting current to cause highly rhythmic neurons to fire in the wrong phase similarly did not affect the averaged DC potential (see Supplemental Figure S1B). Fifth, a good proportion of non-dCIN were also highly rhythmic and thus likely to be CPG neurons. Those that did not exhibit constant DC potentials at rest (96 of 105) did not show such potentials during locomotor-like activity, either (data not shown), discounting the possibility that oscillations in the cL4 motor neurons could alone create a false potential.

In summary, the DC recording is a powerful tool for identifying the synaptic connectivity between spinal cord interneurons and motor neurons during quiescent and locomotor states, a prerequisite for defining a functional role for the interneurons.

Network Organization

The use of spike-triggered averaging of ventral root DC recordings has allowed us to investigate the contribution played by specific interneuron populations to locomotor-like activity and demonstrate how motor output is defined through a mix of inhibition and excitation. The overall organization of the descending CIN system described here differs fundamentally from what has been demonstrated in aquatic vertebrates. In these animals, the locomotor pattern is much simpler, being primarily the alternation between single groups of muscles on the opposing sides of the animal. The CINs involved in swimming CPGs have been described as monosynaptic glycinergic neurons with restricted firing patterns (Soffe et al., 1984; Dale, 1985; Buchanan and Kasicki, 1995). Our data indicate a greater degree of heterogeneity in mammalian CINs with both glutamatergic excitatory and glycinergic inhibitory last-order interneurons as well as polysynaptic innervation of contralateral motor neurons. The described connectivity pattern of the four groups of dCINs is well suited to coordinate the activity between flexor and extensor synergies across the cord as required during normal walking. In this scheme, the functional role of the dCINs in quadrupedal walking CPG is well defined. Although it is not possible for us to ascertain this in the present study, we believe that L2 dCINs also project to caudally located CPG elements (Kjaerulff and Kiehn, 1996; Tresch and Kiehn, 1999), complementing the coordinating role inferred from motor neuron projections. Given the rostrocaudal gradient in rhythmogensis displayed by the lumbar spinal cord (Kjaerulff and Kiehn, 1996; Bertrand and Cazalets, 2002), it is likely that L2 descending inputs, including dCINs, play such a coordinating role.

A limitation in our study is that we have not looked at the projection onto identified L4 extensor and flexor motor neurons. Because of the distance from the cord, it is not possible in the neonatal rodent to record the DC signal in identified peripheral extensor and flexor nerves unlike in chick (Wenner et al., 2000). We have therefore taken advantage of the mixed phase of the motor neurons exiting via the contralateral L4 ventral root to investigate the distribution of dCIN inputs onto both flexor- and extensor-related targets. Thus, during locomotion the phase of L4 ventral root activity was either extensor related, flexor related, or bimodal. There are two possible explanations for this: (1) the same motor neuron population is active in either flexor or extensor phase in different preparations, or (2) different populations of motor neurons (flexors or extensor) were being recorded in different preparations. We find the first explanation unlikely, given that such phase shifts are not seen for any L4 motor neurons when using 5-HT to induce locomotion (Kiehn and Kjaerulff, 1996). Moreover, we never saw any variation in the phase of cL4 relative to iL2 during the course of individual experiments. Finally, the differential firing patterns of the dCINs observed in the flexor and extensor cL4 preparations are hard to reconcile with a model predicting that the same population of motor neurons are activated in different preparation. This would require that *all* four dCIN types terminate onto either flexor *or* extensor motor neurons and that the differential activity of the dCINs in different preparations then defined the motor neurons to be active in either the flexor or the extensor phase. There is no anatomical evidence for such a selective innervation pattern (Eide et al., 1999; Birinyi et al., 2003). Given these arguments, we interpret the variation in L4 phase as reflecting activity in different populations of motor neurons (flexors or extensor) being recorded in different preparations. This allowed us to make assumptions as to the distribution of dCIN inputs onto either flexor or extensor motor neurons as depicted in Figure 6. Importantly, the differential firing patterns of the dCINs observed in the flexor and extensor cL4 preparations suggest that there is a high degree of causality so that the descending L2 drive onto caudal motor neurons and CPG neurons is functional in prescribing whether a flexor or extensor L4 pattern is seen.

Commissural interneurons serve as models for determining the molecular basis of axon guidance at the midline point (Tessier-Lavigne and Goodman, 1996; Kaprielian et al., 2001). Our data provide a functional framework for further investigation of axon targeting following this key intermediate target (see Brittis et al., 2002, and references therein).

Reconfiguration and Plasticity in the Mammalian Locomotor CPG

Connections made by some commissural interneurons were reconfigured when locomotor-like activity was initiated. We have termed these neurons switch cells since they form both monosynaptic excitatory and polysynaptic inhibitory connections onto target extensor motor neurons and undergo a reversal in output sign to cL4 motor neurons.

Given that the monosynaptic excitation is only apparent in the presence of 5-HT, this would suggest that both pathways are subject to differential modulation. The finding that the $GABA_A$ receptor antagonist bicuculline also triggers the switch suggests an intimate interaction between the GABA and 5-HT systems local to the motor neurons. One possibility is that 5-HT acts to suppress GABA release in L4. The net effect of 5-HT application would be to shut down the GABA polysynaptic inhibitory

pathway while simultaneously releasing a monosynaptic excitatory input that is ordinarily suppressed by tonic presynaptic GABA inhibition. There are a number of lines of evidence to support such a hypothesis. First, presynaptic $GABA_A$ receptor-mediated inhibition of spinal premotor neurons has been shown in the lamprey (Alford et al., 1991). Second, GABA has a tonic depressive effect on the rat locomotor network (Bertrand and Cazalets, 1999). Third, tonic GABAergic inputs have been described in higher brain areas (Brickley et al., 2001).

Switch cells also display a long-term facilitation of the excitatory pathway following NMDA- and 5-HT-induced locomotor-like activity. Long-lasting changes in synaptic efficacy, synaptic plasticity, have been described in relation to a number of properties of spinal motor networks (Wolpaw, 1997; Parker and Grillner, 1999; Machacek et al., 2001). Whether there is a requirement for locomotor-like activity or just the presence of NMDA in order to prolong the modulation is as yet unclear and requires further research.

Identification of the switch cells demonstrates that the network connectivity in the mammalian locomotor CPG can be reconfigured by neuromodulators, similar to what has been described for many invertebrate motor systems (Marder and Thirumalai, 2002), and this system provides a good basis for future investigation of reconfiguration and synaptic plasticity in a mammalian, behavioral context.

Flexor and Extensor CPGs Are Differentially Located in the Transverse Plane

By relating the function and anatomical location of dCINs in L2, it became clear that flexor- and extensor-related CINs are differentially distributed in the dorsoventral plane. This high degree of organization of CPG interneuron populations suggest that the flexor and extensor centers in L2 are to some degree anatomically separated. There is no known explanation for this segregation. It is likely, however, that developmental mechanisms underlying dorsoventral interneuron patterning in the mouse spinal cord (Lee and Pfaff, 2001; Sharma and Peng, 2001) are involved in determining the functional and anatomical segregation of dCINs. Ventricular zone P0 and P3 precursor cells characterized by cell-specific transcriptions factors develop into putative V0 and V3 commissural populations, respectively, and migrate to final positions within the ventral horn (Briscoe et al., 1999; Lee and Pfaff, 2001; Moran-Rivard et al., 2001). These separate lineages could define CINs with distinct positions and functions in the cord, similar to what has been described for flexor and extensor motor neurons (Haase et al., 2002; Livet et al., 2002). Future experiments combining intracellular dye labeling from spinal interneurons expressing genetically encoded reporters (Goulding et al., 2002) will be able to test this hypothesis directly.

Conclusions

Neonatal rodents provide an ideal model in which to investigate the development and function of mammalian neuronal networks. Here we describe a component of such networks, one that is necessary for correct crossed coordination of the hindlimbs in mammals. The data provide new insights into the organization and the mechanisms by which interneurons combine to underlie the functioning of a behavior-related neuronal network and provide a vantage point for further characterization of the mammalian locomotor CPG both from a physiological and developmental perspective. Furthermore, an understanding of the spinal cord CPG underlying locomotion is necessary in order to produce effective treatment for spinal cord traumas.

Experimental Procedures

Neonatal (P0–P4) Wistar rats were used for all experiments and the dissection performed as previously described (Kjaerulff and Kiehn, 1996). Briefly, pups were anesthetized with isofluran (in accordance with the stipulations of the local animal care committee and National Institutes of Health guidelines), decapitated, and eviscerated. The isolated spinal cord was pinned ventral side up in a recording chamber superfused with oxygenated (5% CO_2 in O_2) Ringer's solution composed of (mM): 111 NaCl, 3.08 KCl, 25 $NaHCO_3$, 1.18 KH_2PO_4, 1.25 $MgSO_4$, 2.52 $CaCl_2$, and 11 glucose. All experiments were performed at room temperature (20°C–23°C). The protocol for the identification and whole-cell patch recording of caudally projecting dCINs was performed as previously described (Butt et al., 2002) with the exception that the left L4 root (cL4) was left intact and placed in a suction electrode, placed in close proximity to the exit point of the rootlets.

dCIN Spike-Triggered Averaging of DC Ventral Root Recordings

Whole-cell tight-seal recordings of dCINs were performed in current clamp (Axoclamp 2B; Axon Instruments) as previously described (Butt et al., 2002). Patch electrodes (5–8 MΩ) were filled with a solution containing (mM): 138 K-gluconate, 10 HEPES, 0.0001 $CaCl_2$, 5 ATP-Mg, 0.3 GTP-Li. Contralateral cL4 recordings were filtered from DC to 1 kHz. Spike-triggered averaging of the cL4 recording was performed offline using DATAPAC 2000 version 2.41 (Run Technologies Co., Laguna Hills, CA). The threshold for detection of dCIN spikes was normally set at 0 mV. DC potentials could be routinely identified when averaging as little as 50–100 dCIN spikes (see also Brink et al., 1983; Wenner et al., 2000). In most cases, large numbers of spikes were used to determine the precise nature of the DC signal, but these were screened in clusters of 30–50 spikes in order to ensure the signal was not distorted by random large events or artifacts in the DC trace. The calcium dependency of the DC signal was tested using 10% calcium solution, which is known to attenuate synaptic transmission (Tresch and Kiehn, 2000), in which $CaCl_2$ was in part replaced by $MgSO_4$. The latency of the DC potential was determined from the peak of the averaged dCIN action potential to the onset of the potential using DATAPAC. The synaptic latency was calculated based on the estimated time for the dCIN spike to reach the contralateral L4. This was derived from the conduction velocity of the antidromic spike (Butt et al., 2002). To test if the signal was contaminated by signal pickup from the surface of the cord not related to motor neurons, we insulated the tip of the electrode from the cord surface around the exit point of the L4 root with Vaseline in a few experiments ($n = 4$). Application of Vaseline did not attenuate or alter recordings of locomotor activity in the DC trace, nor did it prevent the detection of interneuron spike-triggered DC potentials. These controls suggest that the recorded signals are derived from motor neurons and not from passing fibers in the ventrolateral funiculus. Support for this notion also comes from previously experiments, which showed that a strong synchronous motor neuron DC signal could be recorded from ventral roots when all spike activity was blocked by tetrodotoxin (TTX) (see Tresch and Kiehn, 2000). Furthermore, since the extracellular DC recordings will attenuate fast events more than slow events, motor neuron spikes tend to be filtered out from the signal (see Figure 5) and therefore are unlikely to interfere with the averaging.

Pharmacology of DC Potentials

Strychnine (0.3 μM) and bicuculline (2 μM) were applied at low concentrations and never coapplied in order to minimize spontane-

ous discharge (see Bracci et al., 1996). All short monosynaptic inhibitory DC responses were tested in the presence of CNQX, AP-5. If spontaneous bursting or large-scale oscillations were observed in the ventral root traces, dCIN spikes occurring within that time frame were excluded from the average in order to avoid possible contamination of the DC potential. All pharmacological agents were obtained from Sigma (St. Louis, MO).

Analysis of Locomotor-like Activity

Locomotor-like activity was evoked by perfusion with NMDA (5–8 μM) and 5-HT (5–10 μM). Rhythmic bursting was monitored via a suction electrode placed on the cL2 root (bandpass filtered 100 Hz–1 kHz). Circular statistics were performed to provide a statistical measure of the coupling between dCIN firing and the phase of locomotor-like activity. Analysis was performed on 25 spikes relative to the cL2 ventral root bursts as previously described (Kjaerulff and Kiehn, 1996; Butt et al., 2002). Averaging of the cL4 oscillation relative to the rectified cL2 bursts were performed in DATAPAC.

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